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IDENTIFICATION AND CHARACTERISATION OF MICRORNAS INVOLVED IN CHONDROCYTE DIFFERENTIATION AND OSTEOARTHRITIS

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Purpose: The majority of skeletal bones develop through the process of endochondral ossification. Mesenchymal cells aggregate where future bones will develop. These early chondrocyte cells begin a series of differentiation events, including proliferation, hypertrophy, terminal differentiation, mineralization and programmed cell death. Many of the signalling pathways and transcription factors which control this developmental programme have been established. MicroRNAs (miRNAs) have emerged as a new class of gene expression regulators. MiRNAs are 20-24 nucleotide non-coding RNA molecules that post-transcriptionally regulate gene expression. Little is known about miRNA expression or function in chondrocyte differentiation. In this study we aimed to profile expression of miRNAs in a cell model of chondrocyte differentiation, verify expression of key miRNAs *in vivo* and investigate function.
Methods: The ATDC5 murine embryonic carcinoma cell line was induced to differentiate through chondrogenesis *in vitro*. An Exiqon miRNA microarray was used to profile the expression of all known murine miRNAs across this cell model. Expression of regulated miRNAs was verified in the mouse and chick embryo by *in situ* hybridisation (ISH) using locked nucleic acid probes. To investigate the role of key miRNAs we performed database searches to identify potential targets. 3' UTRs of potential targets were subcloned downstream of a luciferase gene for experimental validation in either SW1353 or C3H10T1/2 cell lines. Expression of key miRNAs was also measured in normal and osteoarthritic cartilage using quantitative RT-PCR.
Results: ATDC5 cell differentiation was verified via GAG staining and measurement of key markers by qRT-PCR (e.g. type II and X collagens). In the expression screen of 609 murine miRNAs, we identified 23 miRNAs which were significantly regulated. Of these, we have pursued miR140 and miR455 for further analyses. The expression of both these microRNAs increased across the time course of differentiation just ahead of hypertrophic markers. We and others have previously shown miR140 to be cartilage-specific. Mir455 is located in an intron of a cartilage collagen gene, Col27a1. ISH shows miR455 is expressed in the developing chick and mouse embryo in the developing skeleton. We (Dalmay) have recently shown that miR140 targets Smad3 to decrease TGFβ signalling. Like miR140, miR455 diminishes Smad-dependent signalling to a $(CAGA)_{12}$-luciferase construct. Furthermore, the expression of both miR140 and miR455 is induced by TGFβ1, TGFβ3 and activinA. Preliminary experiments suggest that mir455 targets Smad2 and the type II activin receptor, acvr2b. Both miR140 and miR455 are also increased in expression in human osteoarthritic cartilage compared to normal.
Conclusions: A number of microRNAs are strongly regulated during chondrocyte differentiation. At least miR140 and miR455 have the potential to modulate Smad signalling in the growth plate and to function as regulators of chondrocyte proliferation and hypertrophy during endochondral ossification. The increase in expression of miR140 and miR455 in human osteoarthritic cartilage may (i) diminish Smad signalling and/or (ii) reflect or regulate the recapitulation of the chondrocyte developmental programme, contributing to pathogenesis.

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CARTILAGE-SPECIFIC MICRORNA-140 REGULATES TISSUE HOMEOSTASIS AND PROTECTS AGAINST OSTEOARTHRITIS-LIKE PATHOLOGY

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Purpose: MicroRNAs (miRNAs) are a class of non-coding RNAs that negatively regulates gene expression by promoting mRNA degradation and/or repressing translation through partial sequence-specific interactions with the 3' untranslated regions (UTRs) of specific mRNA targets. MiRNAs show tissue specific expression patterns, suggesting that these miRNAs play a crucial role in tissue specific physiological processes and also in human diseases. We previously observed that miR-140 has an expression pattern suggestive of a role in chondrocyte differentiation and found that reduced miR-140 expression in human OA cartilage. Reduced miR-140 expression in human OA cartilage prompted us to determine whether miR-140 functions in cartilage homeostasis. The objective of this study was to define the in vivo function of the chondrocyte specific miR-140 in cartilage homoestasis.
Methods: To examine the functions of miR-140, we created a mouse deleted for miR-140 and miR-140 TG mouse, and collected knee joints and chondrocytes from these mice. To examine OA-like pathological changes in articular cartilage, we utilized three different animal models of osteoarthritis: an aging model, a surgical model, and an antigen-induced arthritis (AIA) model. We used a validated histological scoring system based on Safranin O staining and evaluated expression of cartilage related genes by immunohistochemistry. In vitro proteoglycan catabolism was analyzed in cultured femoral head cartilage explants from wild-type, miR-140 TG and miR-140-/- mice. To identify target genes of miR-140, DNA microarray analysis and bioinformatic search were performed. Double-strand (ds) RNA oligos representing mature sequences that mimic endogenous miR-140 were transfected into chondrocytes. Finally, target gene 3' UTR which includes a putative miR-140 binding site was cloned downstream of a luciferase expression vector and the luciferase activity was measured.
Results: OA-like pathology in miR-140-/- mice: Postnatally, miR-140-/- mice manifested a mild skeletal phenotype, with short stature and craniofacial deformities characterized by a short snout and domed skull. First, we tested whether loss of miR-140 affected age-related onset of OA changes, and observed that miR-140-/- mice developed an age-related OA-like pathology. Consistent with observations in the aging OA model, the surgical model also demonstrated that miR-140-/- mice exhibit accelerated OA-like changes in knee joints compared with the wild type. Next, to examine whether miR-140 level in articular chondrocytes affects cartilage sensitivity to experimental challenge, we assessed AIA model. Although miR-140-/- mice showed reduced Safranin O staining, importantly, miR-140 TG mice were resistant to proteoglycan and type II collagen loss compared with wild-type mice.
Target gene of miR-140: Identification of miR-140 target genes could provide new insight into miR-140 function and OA pathogenesis. DNA microarray analysis was performed on RNA samples, and many genes were up-regulated in miR-140-/- chondrocytes compared to wild-type chondrocytes. Adamts-5 emerged as one of strong candidate for miR-140 regulation, and its expression was significantly increased in miR-140-/- mice chondrocytes, and this correlated with increased Adamts-5 protein expression in articular cartilage as seen by immunohistochemistry. Cartilage explants from miR-140-/- mice showed significantly increased proteoglycan release compared to wild-type cartilage. Treatment of chondrocytes with ds-miR140 reduced Adamts-5 expression. Luciferase data indicate that miR-140 directly regulates Adamts-5 expression.
Conclusions: OA-like changes in miR-140-deficient mice can be attributed, in part, to elevated Adamts-5 expression, regulated directly by miR-140. We show that miR-140 regulates cartilage development and homeostasis, and its loss contributes to the development of age-related OA-like changes.

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THE MICRORNAS AS BIOMARKERS SPECIFIC OF KNEE OSTEOARTHRITIS

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Purpose: We are lacking biomarkers predictive of diagnosis and disease progression in OA. MicroRNAs are small RNAs of 21-23 nucleotides able to inhibit gene expression. The objective of this work is to identify the original miRNAs as biomarkers in two different chronic bone and joint diseases, osteoarthritis (OA) and RA.
Methods: Serum samples and fresh blood were obtained from 21 patients with severe knee osteoarthritis (score of Kellgren/Lawrence at least 3/4), 8 RA patients and 10 healthy donors as controls. We performed RNA extraction and qRT-PCR by TaqMan microRNA kit. We analyzed the systemic expression of miRNAs in the 3 groups of patients using microRNA microarrays (Miltenyi). We identified the predictive targets of miRNAs using the DIANA-microT software.
Results: OA patients had a mean age of 71 years, had a mean radiological score of Kellgren/Lawrence of 3.8 and their pain has evolved over 7 years. We identified 37 miRNAs in RA and 18 miRNAs in severe knee osteoarthritis

whose blood expression was altered compared to healthy subjects. In OA, 9 miRNA were up-regulated (including miR-228, miR-574-3p, miR-597) and 9 miRNAs were down-regulated (including miR-150, miR-222, miR-363 and miR-423). None of miRNAs in OA is common with those we found in RA. Potential targets of miRNAs, specifically expressed in severe knee osteoarthritis, appears to be largely involved in the Wnt signaling pathway. In contrast, the miRNAs expressed differently in the blood of our RA patients seem to target the elements of the signaling pathway MAP kinase.
Conclusions: Our results suggest that miRNAs may constitute new biomarkers potential diagnostic interest. In addition, miRNAs could be involved in the pathogenesis of OA. Further work is ongoing in order to assess the pathophysiological and the functional role of these miRNAs in OA.

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STATIN USE IS ASSOCIATED WITH REDUCED INCIDENCE AND PROGRESSION OF KNEE OSTEOARTHRITIS

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Purpose: Besides biomechanical and genetic alterations, the pathogenesis of osteoarthritis (OA) may involve inflammation, vascular alterations and dysregulation of lipid metabolism. Statins are drugs capable of modulating many of these different mechanisms and therefore may have the potential to act as disease modifying drugs for osteoarthritis. In this study we hypothesized that statins decrease incidence and progression of knee and hip OA. To test this hypothesis, we used a large population cohort study.
Methods: 2974 subjects of the Rotterdam Study (a population-based cohort study), aged 55 years and older were included in this study. X-rays of the knee and the hip were obtained at baseline and follow up (mean follow up 6.3 years), and were scored with the Kellgren & Lawrence score (0=absent OA, 1=doubtful OA, 2=mild OA, 3=moderate OA, 4=severe OA, 5= prosthesis). Incidence of OA was defined as a Kellgren & Lawrence score of 0 or 1 and a score of 2 or more at follow up. Progression of OA was specified as a Kellgren & Lawrence score of 1, 2 or 3 and increase of 1 or more. Information on statin use was obtained from detailed computerized pharmacy data. Use of statins was defined as the daily use of 50% or more of recommended dose and this for a period of 100 days or more. Data on potential confounding variables such as age, gender, body mass index, diabetes mellitus, arterial hypertension, peripheral artery disease, bone mineral density and total cholesterol level were collected. A multivariate logistic regression model adjusting for confounding variables was fitted to calculate odds ratios with confidence intervals. Correlations between right and left joints were accounted for with generalized estimating equations.
Results: Osteoarthritis (Kellgren & Lawrence score 2 or more) was present in 546 knees and 323 hips at baseline and in 696 knees and 521 hips at follow up in 1277 men and 1697 women. Overall, 13.2% of subjects were defined as statin users. The adjusted odds ratios for incidence of knee OA in users of statins was 0.40 (95% CI 0.20 - 0.80, p=0.01) and for progression of knee OA 0.47 (95% CI 0.25 - 0.87, p=0.02). The use of statins was neither associated with incidence of hip OA (adjusted odds ratio 0.85, 95% CI 0.54 - 1.34, p=0.48), nor with progression of hip OA (adjusted odds ratio 1.13, 95% CI 0.71 - 1.81, p=0.61).
Conclusions: Statin use is associated with a reduction in incidence and progression of knee osteoarthritis. Randomized clinical trials in a population at risk are needed to examine whether statins could be useful as a treatment for knee OA.

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EIGHTY-SEVEN PERCENT OF 66 ADULT PATIENTS WITH ADVANCED OSTEOARTHRITIS OF THE KNEE SUBJECTED TO TREATMENT WITH INTRA-ARTICULAR INJECTIONS OF HUMAN GROWTH HORMONE AVOID TOTAL KNEE ARTHROPLASTY

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Purpose: Intra-articular injections of growth hormone produce Pre-natal Developmental Healing (PDH). This unique action of growth hormone, proven in mature rabbits, does not involve bleeding, clot formation or the in-pouring of inflammatory cells. This healing is scar-free and produces no fibroartilage. The cascade of PDH first involves the rejuvenation of mature subchondral arteries to form fetal fenestrated capillaries which produce autologous stem cells. These are the same vessels which produce the fetal cartilage skeleton in utero. These stem cells are signaled to form chondrocytes and by the completion of the PDH cascade real articular cartilage with vertical parallel columns of chondrocytes form with arcades at the surface and 100 per cent bonding to host bone. This method is the first to regrow real articular cartilage.
Method: Sixty-six adults with advanced osteoarthritis of the knee were treated with intra-articular injections of Human Growth Hormone (HGH). One-third required arthroscopic debridement and abrasion of eburnated bone on the condyles.
Each knee received from eight to fifteen weekly injections of Human Growth Hormone (HGH). Dosage varied according to the size of the knee. All patients were required to be non weight bearing on the treated side for the duration of the treatment. Patients were required to perform simple exercises at home. Less than one-fourth required physiotherapy.
There were no complications or side effects from the surgery or the injections of human growth hormone (HGH)
Results: Eighty-seven percent of the patients had good to excellent results. Several before and after X-rays of the HGH treated knees will be presented. These X-rays demonstrate an increase of the joint spaces from 2 to 5 mm. Evaluation with the IKDC format will be presented in graph form. There were no infections, complications, side effects, deep vein thrombosis, pulmonary embolism or deaths. The patients who did not respond were no worse. Six per cent went on to have total knee arthroplasty (TKA). the remainder were undecided about having TKA.
Conclusions: A safe, cost-effective alternative to TKA is presented. There were no complications such as those which arise from TKA. The cost of treatment with HGH even including arthroscopic surgery is one-fourth that of TKA. And the additional costs of treating infected TKA are completely avoided. Many orthopedic surgeons are concerned that their livelihood will be adversely impacted by loss of TKA surgery; however, they should consider that there is no need for hospital rounds, or need to treat infections and other serious complications. Insurance companies and Medicare or other government insurance programs can save billions of dollars every year by avoiding costly TKA surgeries at $35,000.00 per TKA.
The author recommends that this HGH treatment, which he named IAGH, be the first choice for treating advanced osteoarthritis of the knee.

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EFFECT OF ROSEMARY EXTRACT AND RELATED FLAVONOID CARNOSOL ON CHONDRO-PROTECTION AND ON THE BONE-CARTILAGE CROSSTALK

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Purpose: The aim of this work was to evaluate the effect of Rosemary extract (P31, Robertet, France) and one of its associated flavonoids, carnosol (Sigma, Buchs, Switzerland) on metabolic functions of chondrocytes and osteoblasts, as well as on the bone-cartilage crosstalk.
Methods: Content of carnosol in rosemary extract P31 was around 7%. Rosemary extract and carnosol efficacy were assessed at various concentrations in the different experiments: 3-25μg/ml and 6nM-30μM, respectively. Normal and Osteoarthritic (OA) human chondrocytes were cultured in alginate beads for 12 days in presence or absence of both compunds. Production of aggrecan (AGG), stromelysin (MMP-3), interleukin (IL)-6 and nitric oxide were analyzed. Isolated human osteoblasts from sclerotic (SC) or non sclerotic (NSC) subchondral bone were cultured for 3 days in presence or absence of both compounds and alkaline phosphatise (AP) activity, interleukin-6 and prostaglandin PGE2 levels were determined. Finally, subchondral osteoblasts coming from NSC or SC areas were incubated with rosemary extract or carnosol for 72h before coculture with OA chondrocytes in alginate beads. After 4 days of co-culture, we analyzed AGG content of the alginate beads, and chondrocytes gene expression of AGG, type II collagen, MMP-3, MMP-13 and osteopontin (OPN).
Results: In both OA and normal chondrocytes, AGG production was significantly increased with 9 μM carnosol. MMP-3 and nitric oxide production was significantly decreased by both compounds, in a dose-dependent manner, while only carnosol significantly decreased the cytokine IL-6 secretion. Regarding osteoblast cultures, AP activity was not affected in the